

<https://helda.helsinki.fi>

Lactobacillus rhamnosus GG in Experimental Oral Biofilms Exposed to Different Carbohydrate Sources

Jiang, Qingru

2018

Jiang , Q , Kainulainen , V , Stamatova , I , Korpela , R & Meurman , J H 2018 , '
Lactobacillus rhamnosus GG in Experimental Oral Biofilms Exposed to Different
Carbohydrate Sources ' , Caries Research , vol. 52 , no. 3 , pp. 220-229 . <https://doi.org/10.1159/000479380>

<http://hdl.handle.net/10138/311448>

<https://doi.org/10.1159/000479380>

unspecified

acceptedVersion

Downloaded from Helda, University of Helsinki institutional repository.

This is an electronic reprint of the original article.

This reprint may differ from the original in pagination and typographic detail.

Please cite the original version.

1 **TITLE**

2 *Lactobacillus rhamnosus* GG in experimental oral biofilms
3 exposed to different carbohydrate sources

4 **AUTHORS**

5 Qingru Jiang, QJ, Department of Oral and Maxillofacial Diseases,
6 University of Helsinki and Helsinki University Hospital, Finland
7 Veera Kainulainen, VK, Medical Nutrition Physiology,
8 Department of Pharmacology, Faculty of Medicine, University of
9 Helsinki, Finland
10 Iva Stamatova, IS, Department of Oral and Maxillofacial Diseases,
11 University of Helsinki and Helsinki University Hospital, Finland;
12 Faculty of Dental Medicine, Medical University of Plovdiv,
13 Bulgaria
14 Riitta Korpela, RK, Medical Nutrition Physiology, Department of
15 Pharmacology, Faculty of Medicine, University of Helsinki,
16 Finland
17 Jukka H Meurman, JHM, Department of Oral and Maxillofacial
18 Diseases, University of Helsinki and Helsinki University Hospital,
19 Finland

20 **RUNNING HEAD**

21 LGG in experimental oral biofilms

22 **KEY WORDS**

23 carbohydrate, probiotic, fluorescent in situ hybridization (FISH),
24 cross-feeding,

25 **CORRESPONDING AUTHOR**

26 Qingru Jiang, Department of Oral and Maxillofacial Diseases,
27 University of Helsinki and Helsinki University Hospital, P.O. Box
28 63 (Haartmaninkatu 8), FI-00014 Helsinki, Finland
29 Phone: +358 440870301 E-mail: qingru.jiang@helsinki.fi

30 **Disclosure Statement**

31 The authors declare no conflicts of interests.

32 **Abstract**

33 Probiotic administration may favour caries prevention as recent research
34 has shown. This in vitro study aimed to investigate the growth of
35 *Lactobacillus rhamnosus* GG (LGG) in experimental biofilms exposed to
36 various carbohydrates, and also to assess its cariogenic potential. Multi-
37 species experimental oral biofilms with/without LGG were grown with a
38 sole-carbohydrate source (fructose/glucose/lactose/sorbitol/sucrose). The
39 viable cells of LGG and structure of biofilms were examined after 64.5h
40 of incubation, and pH values of spent media were measured at 16.5h,
41 40.5h and 64.5h. Fermentation profiles of LGG in biofilm media were
42 assessed with study carbohydrate as the sole energy source. Our results
43 showed that LGG reached higher viable cell numbers with glucose and
44 sucrose in 64.5h multi-species experimental oral biofilms compared to
45 other carbohydrates. When LGG was incorporated in biofilms, no
46 distinct pH changes at all time points were observed under any of the
47 carbohydrates used; the pH values of spent media at each time point were
48 lower when lactose was used, compared to other carbohydrates. The
49 fermentation profiles of LGG in biofilm media were similar to its growth
50 in MRS (no obvious growth with lactose or sucrose). In conclusion, LGG
51 in our in vitro multi-species experimental oral biofilms was capable of
52 surviving and growing well in each carbohydrate source. LGG might not
53 have harmful effects on dental hard tissues. Another finding in our study
54 was that the lowest pH values were observed in the presence of lactose,
55 and the thickest biofilms were in sucrose.

56 **Introduction**

57 Dental caries still remains a global oral health burden worldwide. Caries
58 lesions in enamel and dentin are mainly initiated by the demineralization
59 of the tooth surface through bacterial acid production from sugar
60 [Mayanagi et al., 2017]. Sucrose, fructose, and glucose are considered
61 the most important sugars/carbohydrates in caries development and
62 progression [Marsh, 2003; Selwitz et al., 2007]. Acid-producing bacteria
63 commonly associated with dental caries are *Streptococcus mutans*
64 [Forssten et al., 2010], lactobacilli [Jiang et al., 2015], and *Actinomyces*
65 [Xiao et al., 2016], which are inherent residents of oral biofilms
66 developing on tooth surface. In the last decade, an increasing number of
67 studies have shown great interests in the prevention of caries with the
68 usage of probiotics [Laleman and Teughels, 2015; Jorgensen et al.,
69 2016].

70 Probiotics are ‘live microorganisms that, when administered in adequate
71 amounts, confer a health benefit on the host’ [Hill et al., 2014]. Among
72 the probiotics strains *Lactobacillus rhamnosus* GG (ATCC 53103, LGG)
73 is one of the most documented and widely used probiotic strains in the
74 world. Beneficial effects of LGG in general have been documented in
75 various clinical trials, including studies on diarrhoea, allergy, and liver
76 diseases [Floch et al., 2015].

77 A fair number of clinical trials also suggest that both short- and long-
78 term intake of probiotic could reduce *S. mutans* counts in saliva and/or
79 plaque [Meurman et al., 1995; Näse et al., 2001; Aminabadi et al., 2011;
80 Laleman et al., 2014; Tehrani et al., 2016]. However, a more pronounced
81 beneficial effect of saliva-derived lactobacilli was observed in subjects
82 without caries experience rather than in individuals with arrested or
83 active caries lesions [Simark-Mattsson et al., 2007]. There is still paucity
84 of evidence to establish relationship between probiotic administration
85 and decayed/missing/filled teeth (DMFt) scores [Simark-Mattsson et al.,
86 2007; Gruner et al., 2016; Tehrani et al., 2016]. In addition, the safety of
87 probiotic use in the oral cavity has been a controversial topic. The genus
88 of *Lactobacillus* is known for their acidophilic properties, which in light
89 of the aetiology of dental caries may impose an inherent risk to dental

90 hard tissues. In the other hand, probiotics have proven safe both in vitro
 91 and in vivo studies [Snydman, 2008]. Pham et al. [2011] have suggested
 92 that LGG had no significant effect on cariogenic potential of a complex
 93 saliva-derived biofilms. However, Schwendicke et al. [2014a; 2014b]
 94 have reported that LGG and *Bifidobacterium* BB12 were found to
 95 demineralize both enamel and dentin, and LGG even induced increased
 96 demineralization compared to *S. mutans* mono-species biofilm alone.
 97 Although there are limited aspects of positive effects for caries
 98 prevention and insufficient safety studies, probiotics significantly
 99 increased the chance of reducing *S. mutans* [Gruner et al., 2016] and
 100 mutans streptococci are major pathogens of dental caries [Takahashi and
 101 Nyvad, 2011], which leads the probiotic use in caries prevention as a hot
 102 topic. Accordingly, the inhibitory activity of probiotic against common
 103 oral pathogens (*S. mutans*, *Candida albicans*, *Streptococcus sanguinis*)
 104 has been also tested in vitro [Soderling et al., 2011; Jiang et al., 2015;
 105 Wu et al., 2015; Jiang et al., 2016] and its fermentation profiles have
 106 been a subject of studies [Hedberg et al., 2008; Douillard et al., 2013].
 107 However, there is limited evidence about the ability of LGG to establish
 108 itself in the human mouth and to integrate and interact with oral biofilms.
 109 Studies in this regard are needed and would contribute towards
 110 understanding the mechanisms behind beneficial effects of probiotics
 111 from the oral health perspective.
 112 Our previous results affirmed that probiotic LGG was able to integrate
 113 with experimental oral biofilms in vitro and differently affected the
 114 growth of tested cariogenic strains [Jiang et al., 2016]. In the present
 115 study, a sequel to our previous work, our aim was to investigate the
 116 growth of LGG in experimental oral biofilms under various carbohydrate
 117 conditions and to evaluate its potential risk for dental hard tissues in
 118 terms of pH alterations to growth environment.

119 **Materials and Methods**

120 **Strains, growth conditions, and inoculum preparation**

121 LGG, from Valio Ltd., Helsinki, Finland was used as the probiotic strain
 122 in our study. Biofilms in control group (5SP) were built with the pool of
 123 five species of oral bacterial/yeast strains: *C. albicans* ATCC 10231, *S.*

124 *mutans* ATCC 27351, *S. sanguinis* ATCC 10556, *Aggregatibacter*
 125 *actinomycetemcomitans* ATCC 43718, and *Fusobacterium nucleatum*
 126 ATCC 25586. Group of 5SP with LGG (5SP+LGG) was the study group.
 127 All the strains were maintained as frozen stock at -80°C in 20% skim
 128 milk (Difco™, BD, Becton, Dickinson and Company, Sparks, MD,
 129 USA). Before each experiment, strains were cultivated twice on
 130 respective agars (details are given in Table 1). Pure colony of each strain
 131 was inoculated in 5 mL corresponding cultivation broth, and cultivated
 132 anaerobically overnight at 37°C.
 133 Bacterial and yeast strains were harvested by centrifugation for 10 min at
 134 3,000 × g, at room temperature, washed three times with 5 mL 0.9%
 135 NaCl and re-suspended in biofilm medium base (BMB, biofilm medium
 136 sugar free) adapted from Lemos et al. [2010]. The cell suspensions were
 137 adjusted to an OD₄₉₀ of 0.130±0.010 (similar to McFarland standard No.
 138 1. The cell concentrations of suspensions were 1.64×10⁸ cells/mL for
 139 LGG, 3.33×10⁷ cells/mL for *C. albicans*, 7.53×10⁸ cells/mL for *S.*
 140 *mutans*, 3.31×10⁸ cells/mL for *S. sanguinis*, 4.44×10⁹ cells/mL for *A.*
 141 *actinomycetemcomitans*, and 1.72×10⁸ cells/mL for *F. nucleatum*) by a
 142 spectrophotometer (Multisan Plus, Labsystems, Helsinki, Finland,
 143 measured by 200 µL each well in 96-well plate). Aliquots of strain
 144 suspensions were then pooled for control group (5SP) and study group
 145 (5SP+LGG), according to the group setup.

146 **Preparation of biofilms**

147 Biofilms were grown on saliva-coated hydroxyapatite (HA) discs
 148 (Clarkson Chromatography Products, Inc., South Williamsport, PA,
 149 USA). The discs were 7.0 mm in diameter and 1.8 mm high. The HA
 150 discs were placed in a vertical position in disc holders bent from
 151 orthodontic wire according to Lemos et al. [2010] with minor
 152 modifications. The holders and the HA discs were autoclaved after
 153 assembling.
 154 To allow formation of a salivary pellicle, each HA disc was placed in a
 155 well of a sterile 24-well polystyrene cell culture plate, fully immersed
 156 and incubated with 1.8 mL of processed saliva and by gentle shaking for
 157 4h at room temperature. Whole saliva was collected from 21 healthy

648 **Table 1.** Strains and the growth conditions.

Strain	Origin	Agar/Broth	Growth conditions
<i>Lactobacillus rhamnosus</i> GG ATCC 53103 (LGG)	Valio Ltd., Helsinki, Finland	de Man, Rogosa and Sharpe (MRS)	24h, 37°C, 5% CO ₂
<i>Candida albicans</i> ATCC 10231	American Type Culture Collection (ATCC)	Sabouraud	24h, 37°C, air
<i>Streptococcus mutans</i> ATCC 27351	ATCC	Brain Heart Infusion (BHI)	24h, 37°C, 5% CO ₂
<i>Streptococcus sanguinis</i> ATCC 10556	ATCC	BHI	24h, 37°C, 5% CO ₂
<i>Aggregatibacter</i> <i>actinomycetemcomitans</i> ATCC 43718	ATCC	BHI	24h, 37°C, 5% CO ₂
<i>Fusobacterium nucleatum</i> ATCC 25586	ATCC	Brucella	48h, 37°C, in anaerobic condition (mixture of 0.2% O ₂ , 5% CO ₂ , 9.9% H ₂ , 84.9% N ₂)

649

158 volunteers after their informed consent (men, n=10, women, n=11; mean
159 age 35±8). Pregnancy, gingival bleeding, history of antibiotic
160 administration in the past 2 weeks, and eating, drinking and oral hygiene
161 procedures 1.5 hour prior saliva collection were the main exclusion
162 criteria. The processed saliva was prepared and pasteurized according to
163 Guggenheim et al. [2001]. The efficacy of pasteurization was assessed by
164 plating processed saliva samples onto Brucella agar (BBL™, BD,
165 Becton, Dickinson and Company, Sparks, MD, USA, with vitamin K3 10
166 ug/mL, hemin 5 ug/mL, and 5% defibrinated horse blood from bio
167 TRADING, Mijdrecht, the Netherlands), and cultivated either aerobically
168 or anaerobically for 3 days, until no colonies were observed.
169 After the saliva-coating step, HA discs were transferred to a new 24-well
170 plate containing 2.5 mL biofilm culture medium and 0.3 mL pooled
171 strains in each well. Six biofilm culture media were used in this study,
172 namely BMB with water (BM-negative), with fructose (BM-fructose),
173 with glucose (BM-glucose), with lactose (BM-lactose), with sorbitol
174 (BM-sorbitol), and with sucrose (BM-sucrose). The concentration of
175 carbohydrate was 3.6 g/L (i.e. 20 mM glucose/fructose/sorbitol or 10
176 mM lactose/sucrose). Then the plates with HA discs and broth media
177 were incubated anaerobically at 37°C for 64.5 h in dark. Broth media
178 were renewed at 16.5h and 40.5h as the following steps: the discs were
179 first washed by dipping twice into 2.8 mL physiological saline and then
180 transferred to a new 24-well plate containing 2.8 mL fresh broth media
181 per well.

182 **Examination of LGG cells**

183 After 64.5h cultivation and two dip washes in physiological saline, each
184 HA disc was transferred into a sterile 50 mL polypropylene tube
185 containing 5 mL of physiological saline at room temperature, and
186 vortexed (by Vortex-Genie® 2 mixer, Scientific industries, Inc, Bohemia,
187 N.Y., USA) vigorously for 2 min, and sonicated (by Wagner instrusonic,
188 PS-Terä Oy, Lahti, Finland, 90/180 watts) for 5 sec at room temperature.
189 Serial dilutions of the sonicated cells were cultivated on de Man, Rogosa
190 and Sharpe (MRS; Lab M Ltd, Bury, UK) agar plates at 37°C in 5% CO₂

191 for 72h. Colony forming units (CFU) of LGG were counted based on its
192 colonial morphology on MRS.

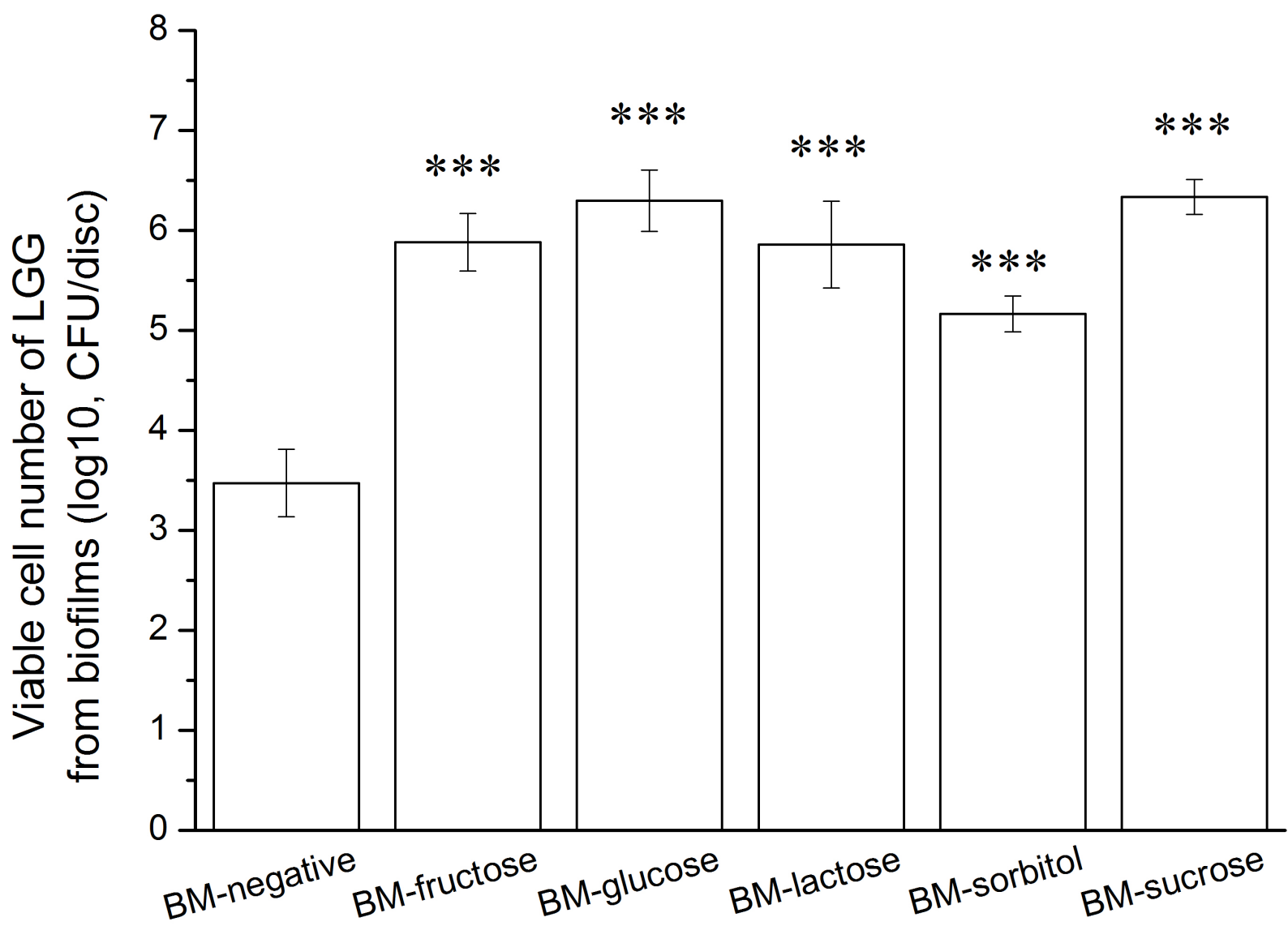
193 **Measurement of pH values of spent culture media**

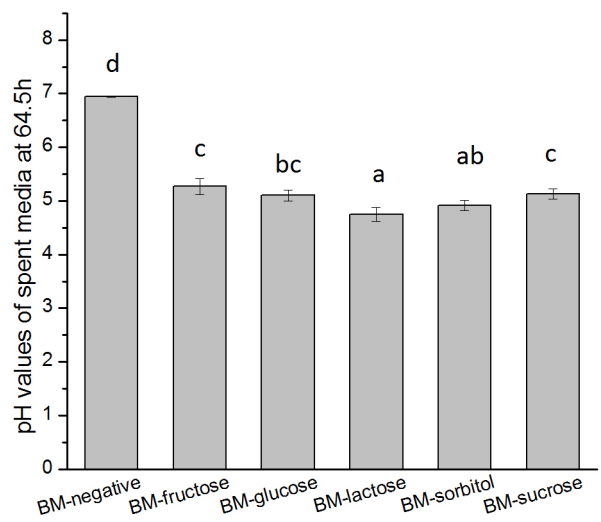
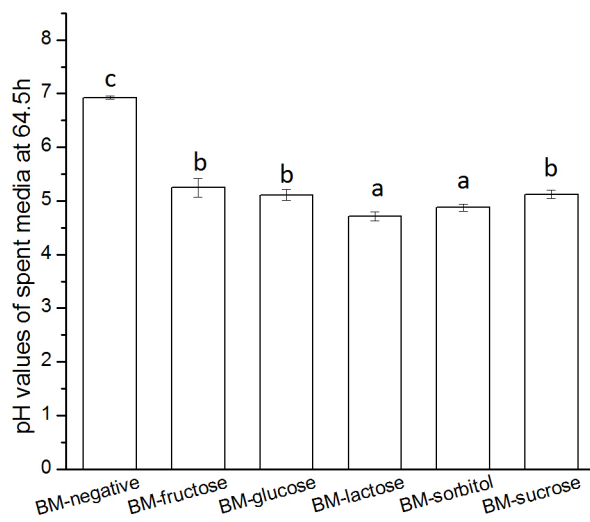
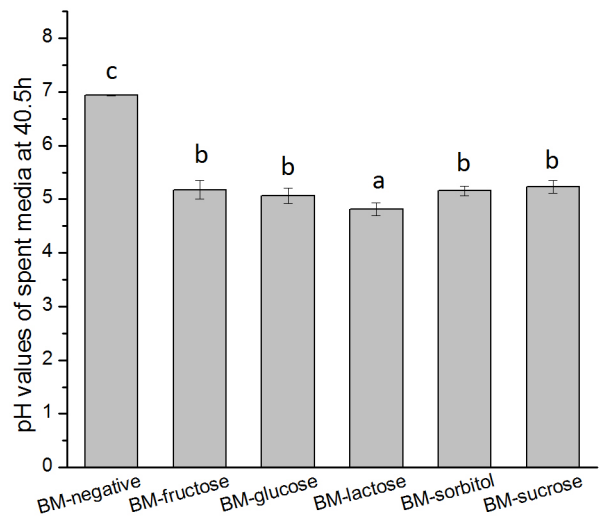
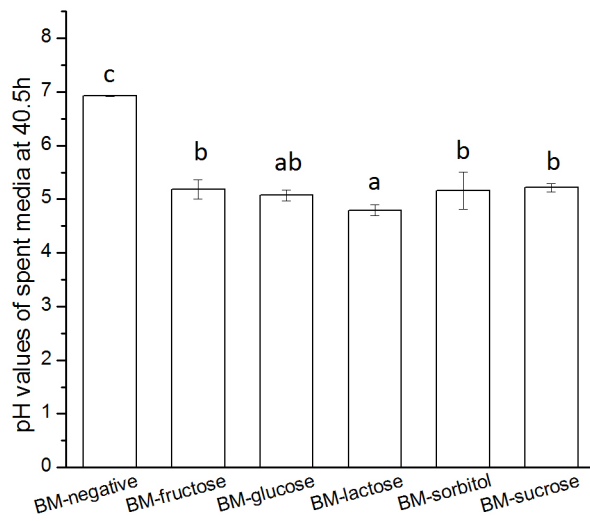
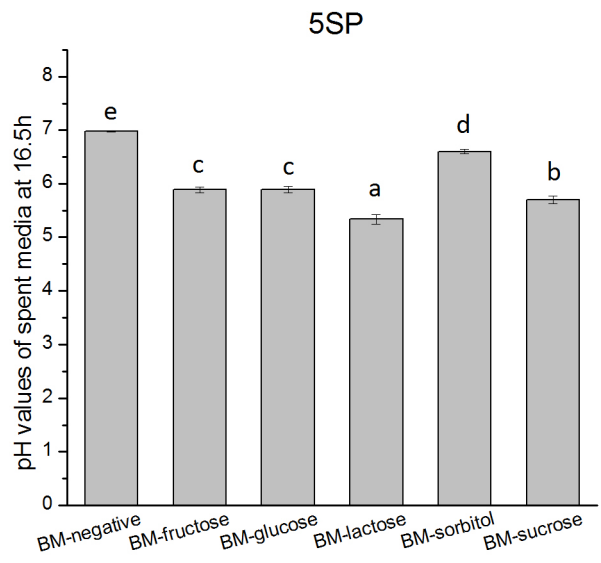
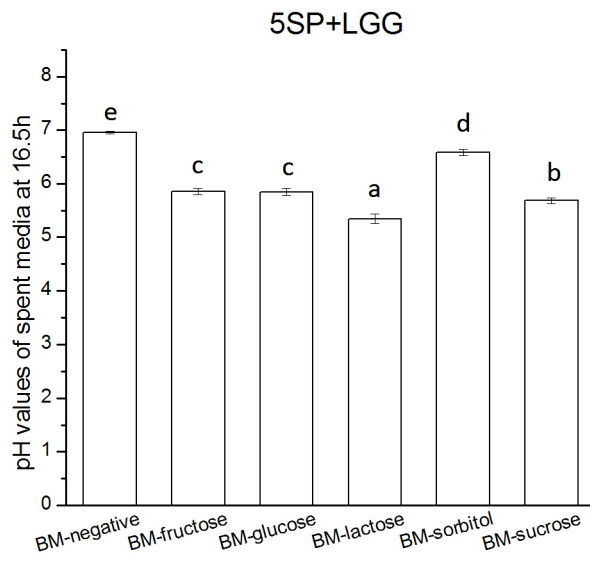
194 The pH of spent media was measured with a pH meter (pH 1000 L,
195 pHenomenal®, VWR International, Rador, PA, USA) at all three time
196 points, when the HA discs were transferred into fresh media or
197 physiological saline and when the spent media were replaced. The spent
198 media were centrifuged for 10 min, $3,000 \times g$ prior to pH measurement
199 from the supernatant.

200 **Structural analysis of biofilms**

201 The biofilm structure was analysed with the method of fluorescence in
202 situ hybridization (FISH).

203 For FISH analysis, the staining was performed mainly according to the
204 protocol established by Thurnheer et al. [2004]. In short, 64.5h biofilms
205 were fixed immediately with 4% (w/v) paraformaldehyde for 1h at 4°C,
206 permeabilized for 30 min at 37°C by exposure to the mixture (46200
207 U/ml or 1 mg/ml lysozyme, 98 mM Tris/HCl, 5 mM EDTA, pH 7.5.
208 Extra 100 U/mL mutanolysin was added for Group 5SP+LGG), pre-
209 hybridized in hybridization buffer (0.9M NaCl, 20mM Tris/HCl, 30%
210 Formamide, 0.01% SDS) at 46°C for 15 min, and followed by
211 hybridization for 3h with fluorescently labelled oligonucleotides
212 (Lcas467-Cy3 probe binds LGG: 5'-CCGTCACGCCGACAACAG-3'
213 [Ardita et al., 2014], and MUT590-Cy5 probe binds *S. mutans*: 5'-
214 ACTCCAGACTTTCCTGAC-3' [Quevedo et al., 2011]). After
215 hybridization, biofilms were washed twice in wash buffer (20mM
216 Tris/HCl, 5mM EDTA, 102 mM NaCl, 0.01% SDS) and stained with
217 Hoechst (to stain the rest of the strains= *S. sanguinis* + *C. albicans* + *A.*
218 *actinomycetemcomitans* + *F. nucleatum*) for 5min in dark.
219 Afterwards all the samples were embedded in Mowiol [Thurnheer et al.,
220 2006] overnight at room temperature and were examined with an
221 inverted Confocal Laser Scanning Microscopy (CLSM) Leica SP8 (Leica
222 Microsystems GmbH Wetzlar, Germany). CLSM images were obtained
223 with a $\times 40$ water immersion objective. Each biofilm was scanned at
224 randomly selected areas as a series of vertical optical sections, each

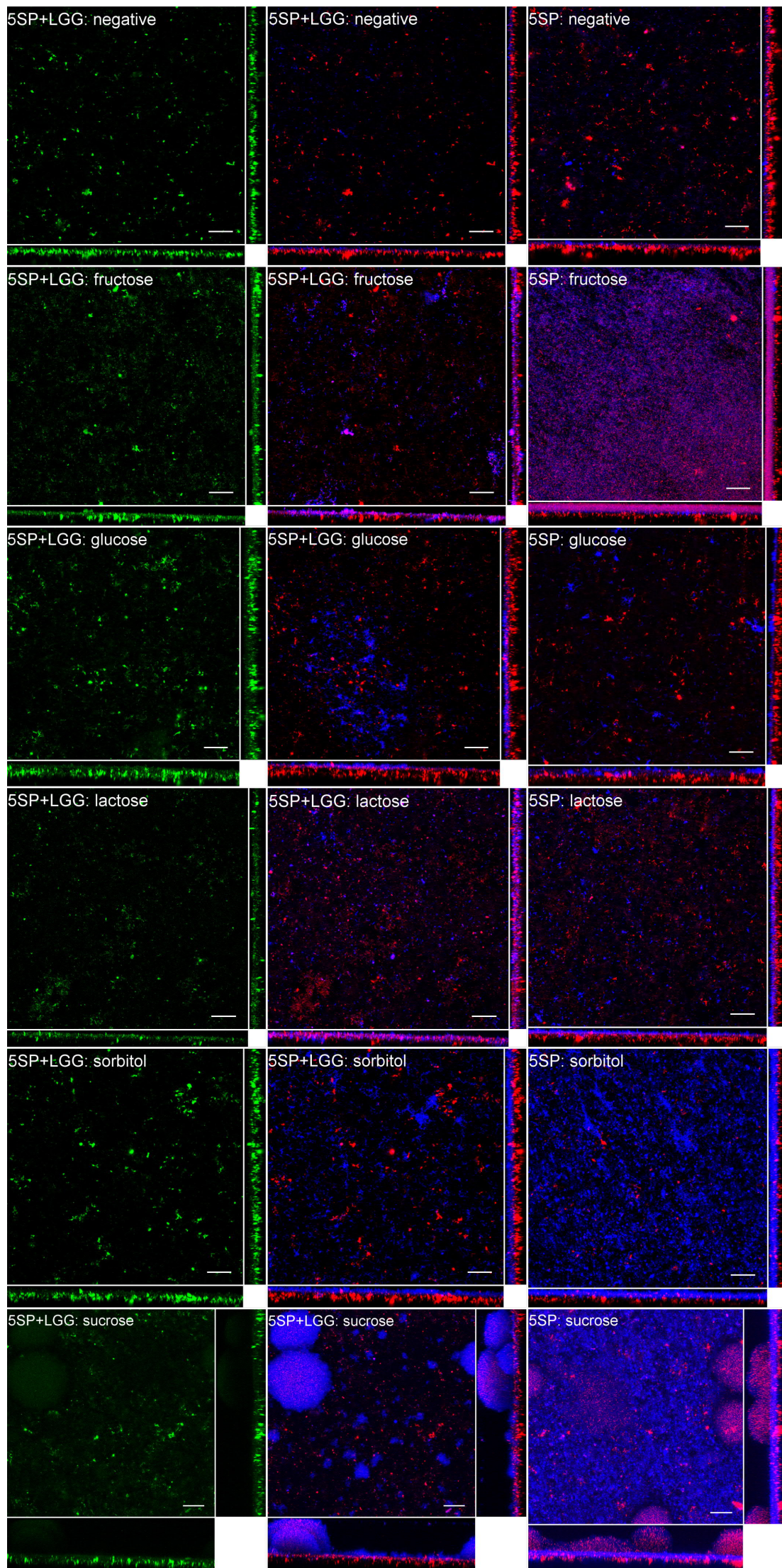




A

B

C



225 section was 0.50 μm thick. Digital images were processed with Fiji
226 [Schneider et al., 2012].

227 **Fermentation profiles of LGG in biofilm medium with sole** 228 **carbohydrate source.**

229 The overnight cultures of LGG were harvested by centrifugation for
230 10min at $3,000 \times g$, at room temperature, washed three times with 5 mL
231 0.9% NaCl and re-suspended in BM-negative medium. The suspensions
232 were adjusted to an optical density at 490 nm (OD_{490}) of 0.360 ± 0.010 .
233 The adjusted suspension (200 μL) was inoculated into 5mL aliquots of
234 BM-negative, fructose, glucose, lactose, sorbitol, or sucrose media,
235 respectively, and cultivated in 5% CO_2 at 37°C. The growth was
236 measured by observing the changes of OD_{490} at 0, 4, 6, 20, 24 and 48h
237 incubation.

238 **Statistical analysis**

239 Data are shown as means \pm standard deviations. Statistical analyses were
240 performed with IBM SPSS Statistics version 22 for Windows. One-way
241 ANOVA and Dunnett's test were used to determine statistical
242 significance in Figure 1, and Duncan's test in Figure 2. A difference was
243 deemed significant at $P < 0.05$ or $***P < 0.001$. Log10 transformation of
244 the viable cell numbers was made before the statistical analysis.

245 **Results**

246 **Growth of LGG in biofilms**

247 The viable cell numbers of LGG in 64.5h experimental oral biofilms
248 cultured with the five sole-carbohydrate media are presented in Figure 1.
249 LGG was able to use all the supplemented carbohydrates for growth and
250 viable cells of LGG were detected in all the biofilms, including the
251 negative control group. LGG grew to higher number when the
252 carbohydrate source was glucose ($2.33 \pm 1.60 \times 10^6$ CFU/disc) and sucrose
253 ($2.29 \pm 0.99 \times 10^6$ CFU/disc) compared with the other carbohydrate
254 sources. These numbers were significantly ($P < 0.001$) higher than that in
255 the negative control group ($3.54 \pm 2.18 \times 10^3$ CFU/disc). Among the study
256 groups, the lowest viable cell number of LGG was measured when
257 sorbitol was used ($1.55 \pm 0.58 \times 10^5$ CFU/disc). In the presence of lactose

258 and fructose, the numbers of LGG were $9.67 \pm 8.12 \times 10^5$ CFU/disc and
259 $8.88 \pm 6.39 \times 10^5$ CFU/disc, respectively.

260 The highest viable cell numbers of LGG in the experimental oral
261 biofilms were observed in the presence of glucose, followed by sucrose,
262 lactose, fructose, sorbitol and negative control.

263 **pH values of spent media**

264 The pH values of spent media (Figure 2) were measured when new broth
265 media were replaced or at the end of cultivation, i.e. at 16.5h, 40.5h and
266 64.5h, respectively.

267 The pH values of spent media at 16.5h were above 5. The presence of
268 LGG did not clearly change the pH values of the spent media when
269 comparing the groups of 5SP+LGG and 5SP which had been cultivated
270 with each carbohydrate studied and at each time point.

271 The pH values in the carbohydrate-supplemented groups were
272 significantly lower than that in the negative group ($P < 0.05$).

273 The lowest pH values in 5SP+LGG and 5SP groups at all time points
274 (respectively at 16.5h: 5.34 ± 0.09 and 5.34 ± 0.09 , at 40.5h: 4.79 ± 0.10 and
275 4.81 ± 0.12 , at 64.5h: 4.72 ± 0.09 and 4.75 ± 0.14) were measured from the
276 subgroup BM-lactose.

277 **Biofilms structure**

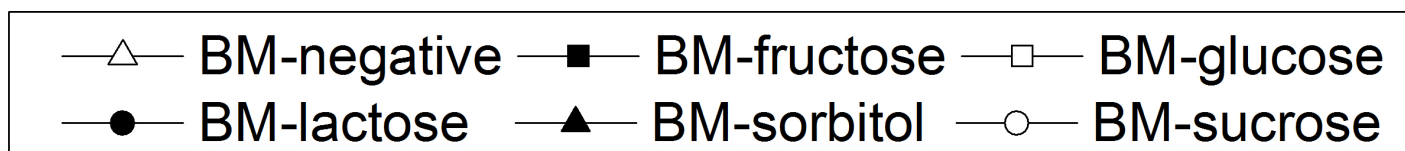
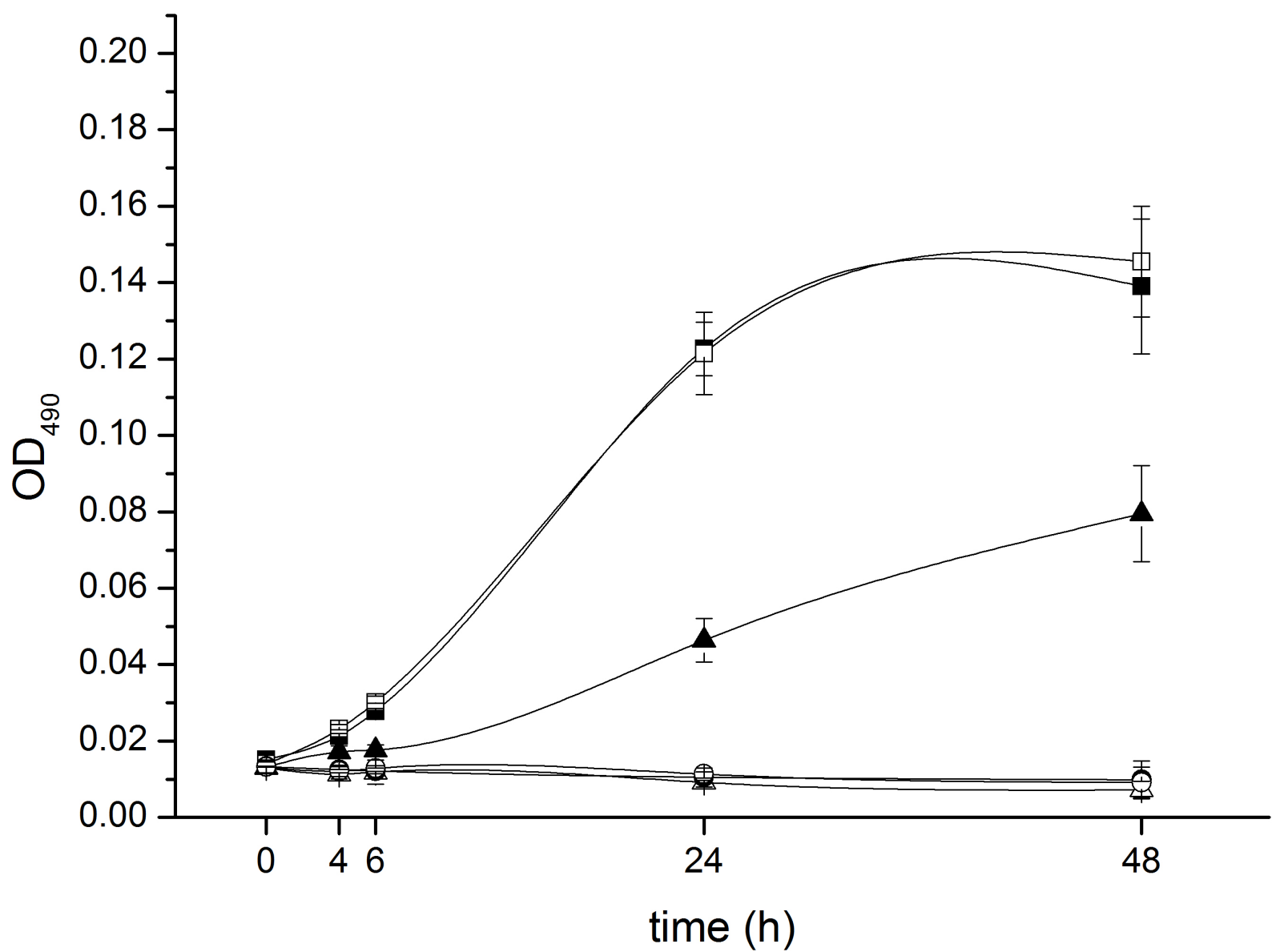
278 All the microbes in 64.5h biofilms grew out as layer structures (Figure
279 3), and hemispherical shape structures were observed in the presence of
280 sucrose.

281 From column A, LGG was able to be detected in 64.5h biofilms of group
282 5SP+LGG under all the tested carbohydrate conditions.

283 Comparing columns B and C with each carbohydrate, less microbes
284 (both red and blue channels) were adhered and developed in group
285 5SP+LGG than in group 5SP. Also the first layer of biofilms of group
286 5SP was mainly composed of *S. mutans*, but this layer in group
287 5SP+LGG was mostly mixed up with the rest of the strains.

288 **Planktonic cell growth**

289 In order to compare the growth of LGG in the experimental oral biofilms
290 and as planktonic cells, we also tested the fermentation profiles of LGG
291 in biofilm broth media with the five sole-carbohydrates in 48h. Figure 4



shows that at the end of cultivation LGG grew to highest optical density in subgroups BM-glucose and BM-fructose, and higher in BM-sorbitol. No obvious increases of optical density were found in BM-negative, BM-lactose, and BM-sucrose, respectively. The highest growth of LGG in the biofilm broth media was in the presence of glucose, followed by fructose, sorbitol, negative control, and lactose, while least growth was observed in the presence of sucrose.

Discussion

This in vitro study aimed to investigate LGG growth in experimental oral biofilms simulating oral conditions and, secondly, to evaluate the potential of this probiotic strain in decreasing pH in its environment in the perspective of dental caries. We built 64.5h multi-species experimental oral biofilms cultivated with fructose, glucose, lactose, sorbitol, and sucrose. Our results demonstrate that LGG can grow to higher viable cell numbers with glucose and sucrose in these multi-species biofilms compared to the other carbohydrates. Furthermore, the addition of LGG did not decrease the pH values in the experimental model systems.

The growth of LGG in the multispecies experimental oral biofilms was different from its growth in mono-species biofilms or as planktonic cells. We found that LGG in our study was able to survive and grow well in a wider spectrum of carbohydrate sources. The growth of LGG as planktonic cells in the biofilm broth media was similar to that in MRS [Jiang et al., 2015], showing low or no capability to utilize sucrose or lactose. But LGG in the multispecies experimental oral biofilms did show better growth in the presence of sucrose or lactose. In the study of Hedberg et al. [2008] LGG was found to ferment sucrose or lactose only after 48h and 72h cultivation. Another possible reason to explain its growth in sucrose and lactose in our experiment is that *S. mutans* [Moye et al., 2014], *S. sanguinis* [Tanzer et al., 1971; Yamada et al., 1985] and/or *C. albicans* [Binkley et al., 2014] when present in the biofilms could hydrolyse these two carbohydrates to fructose, glucose, and galactose. Then fructose and glucose could be easily utilised by LGG leading to higher viable cell numbers observed.

One of our important findings is that the growth of LGG in sucrose or lactose with cross-feeding is here demonstrated. Whenever one organism uses metabolites produced by another organism as energy or nutrient sources, it is called cross-feeding [Estrela et al., 2012]. A recent study from Pan et al. [2016] has demonstrated that cooperative cross-feeding between different bacterial species is favoured in structured environments such as bacterial biofilms, suggesting that this type of interactions might be common in natural bacterial communities. Apparently, the nutritional interaction in the present study was beneficial regarding the growth of LGG.

In addition, when glucose was the sole carbohydrate source, the viable cell number of LGG in the multispecies experimental oral biofilms ($2.33 \pm 1.60 \times 10^6$ CFU/disc) was more than seven times higher than the viable cell number of LGG in its mono-species biofilms ($3.16 \pm 1.80 \times 10^5$ CFU/disc) [Jiang et al., 2016]. This finding could be explained by the theory that microbial consortia actively attempt to become polymicrobial in order to gain resistance and better survival ability [Wolcott et al., 2013].

In our series LGG showed no cariogenic potential since the pH values of the spent media at 16.5h were not decreased when LGG was co-cultured. These pH values did not drop close to or below the critical levels for dental enamel demineralization (i.e. pH 5.2-5.5) [Dahlén et al., 2012]. This phenomenon in the present study might be explained by three possible ways: 1) The biofilm medium contained 68.5 mmol/L phosphate, which prevented any drastic pH change; 2) the acids produced by LGG were utilized by the other micro-organisms and thus did not affect the environment; and 3) LGG cells comprised only a small part in the biofilms, so the acids generated by them did not decrease the pH in the whole model system.

Commercial probiotic products are now widely available, so safety issues are raising up. Numerous in vitro and in vivo studies have been published to address the consumption of probiotics from various perspectives. For example, Hibberd et al. [2014] have reported that in a 28-day clinical trial, LGG was safe in healthy adults aged 65 years and

360 older with no serious adverse events. And a two-week consumption of
361 *Lactobacillus reuteri* and LGG appeared not to influence the
362 acidogenicity of plaque of young adults [Marttinen et al., 2012].
363 Stamatova et al. [2007] have proved that intake of *Lactobacillus*
364 *bulgaricus* strains is not anticipated to exert any deleterious effects on
365 the regulatory enzymes and structure of the host extracellular matrix.
366 However, some reports do not agree with the above conclusions.
367 Probiotics strains, for example, *Lactobacillus salivarius* strains, LGG,
368 BB12 have been reported to show ability to induce caries and mineral
369 loss in vivo and in vitro [Matsumoto et al., 2005; Pham et al., 2009;
370 Schwendicke et al., 2014a; Schwendicke et al., 2014b]. These
371 contradictory reports urge more relevant future studies to clarify the
372 safety issue. Meanwhile the effects of probiotics are strain-dependant, it
373 is crucial to select no cariogenic risk strains as oral probiotics.
374 In the present study, it was interesting to find out that the pH values of
375 the spent media were lowest in the presence of lactose. Lactose is one of
376 the major sugars in dairy products and its fermentation can potentially
377 demineralize dental hard tissues. Traditionally, sucrose is regarded the
378 most cariogenic sugar [Boonyanit et al., 2011]. In our study sucrose in
379 the growth medium indeed resulted in low pH values of the spent media
380 but not as low pH values as lactose. This finding might be used to advise
381 consumers to choose lactose-free probiotic products. However, it should
382 be kept in mind that milk, for example, has a strong buffering capacity
383 [Salaun et al., 2005]. Thus, studies in clinical setting are called for before
384 drawing any further conclusion in this respect.
385 Although lactose led to a lowest pH, sucrose resulted in thicker biofilms,
386 which agrees with and proofs that sucrose is the most cariogenic sugar
387 [Gupta et al., 2013]. And the biofilms' structure implies that *S. mutans*
388 colonized the saliva-coated HA surface earlier than the rest of the strains
389 and the addition of LGG affected the adherence of *S. mutans*, which are
390 consistent with previous observations [Li et al., 2004; Jiang et al., 2016].
391 These results all prove that this biofilm model is effective and repeatable.
392 One of the strengths of this study is to involve multi-species to build
393 biofilms to mimic a complex ecosystem, but it is also a limitation.

394 Because the dynamic oral cavity contains far more species to form
395 various microbial communities, and there are great inter-individual
396 variations [Sato et al., 2015]. Also the tested strains are all reference
397 strains. Hence the findings in this study need to be further confirmed and
398 ideally in a clinical setting.

399 Within the limitations of this study, LGG in our in vitro multi-species
400 experimental oral biofilms was capable of surviving and growing well
401 with each of the studied carbohydrate sources. The lowest pH values
402 were observed in the presence of lactose.

403 **Acknowledgements**

404 Many thanks to Saija Perovuo for her help in the laboratory. Dr. Sok-Ja
405 Kim Janket from Boston University is thanked for her guidance with the
406 statistical analyses. Dr. Thurnheer Thomas from University of Zurich is
407 thanked for the FISH protocol. Biomedicum Imaging Unit (BIU) from
408 Faculty of Medicine, University of Helsinki is thanked for the technical
409 assistance and microscopy services. Professor Seppo Salminen from
410 University of Turku is thanked for his critical comments in the
411 preparation of the manuscript. The study was funded by the Department
412 of Oral and Maxillofacial Diseases Scientific Research Laboratory,
413 University of Helsinki. QJ was funded by the China Scholarship Council
414 (201206310016) and Selma and Maja-Lisa Selander's Fund for Research
415 in Odontology. VK was funded by the Academy of Finland (grant No.
416 285632). The funders had no role in the study design, data collection and
417 analyses, decision to publish, or preparation of the manuscript.

418 **Author Contributions**

419 All authors conceived and designed the experiments. QJ performed the
420 experiments, analysed the data and drafted the manuscript. VK, RK, IS,
421 JHM revised the manuscript. All authors read and approved the final
422 manuscript.

423

424 **References**

- 425 Aminabadi NA, Erfanparast L, Ebrahimi A, Oskouei SG: Effect of
426 chlorhexidine pretreatment on the stability of salivary
427 lactobacilli probiotic in six- to twelve-year-old children: a
428 randomized controlled trial. *Caries Res* 2011;45:148-154.
- 429 Ardita CS, Mercante JW, Kwon YM, Luo LP, Crawford ME, Powell DN,
430 Jones RM, Neish AS: Epithelial adhesion mediated by pilin SpaC
431 is required for *Lactobacillus rhamnosus* GG-induced cellular
432 responses. *Appl Environ Microbiol* 2014;80:5068-5077.
- 433 Binkley J, Arnaud MB, Inglis DO, Skrzypek MS, Shah P, Wymore F,
434 Binkley G, Miyasato SR, Simison M, Sherlock G: The *Candida*
435 Genome Database: the new homology information page
436 highlights protein similarity and phylogeny. *Nucleic Acids Res.*
437 42 (Database issue): D711-6. URL:
438 [http://pathway.candidagenome.org/CALBI/NEW-](http://pathway.candidagenome.org/CALBI/NEW-IMAGE?type=PATHWAY&object=PWY-621)
439 IMAGE?type=PATHWAY&object=PWY-621; in., 2014.
- 440 Boonyanit T, Sroisiri T, Doan Minh T: Fermentation of various sugars and
441 sugar substitutes by oral microorganisms. *Asian Pac J Trop Med*
442 2011;1:S258-S260.
- 443 Dahlén G, Fiehn N-E, Olsen I, Dahlgren U: Oral microbiology and
444 immunology. Copenhagen, Denmark, Munksgaard Danmark,
445 2012.
- 446 De Filippis F, Vannini L, La Stora A, Laghi L, Piombino P, Stellato G,
447 Serrazanetti DI, Gozzi G, Turroni S, Ferrocino I, Lazzi C, Di Cagno
448 R, Gobbetti M, Ercolini D: The same microbiota and a potentially
449 discriminant metabolome in the saliva of omnivore, ovo-lacto-
450 vegetarian and vegan individuals. *PLoS One* 2014;9:e112373.
- 451 Douillard FP, Ribbera A, Jarvinen HM, Kant R, Pietila TE, Randazzo C,
452 Paulin L, Laine PK, Caggia C, von Ossowski I, Reunanen J,
453 Satokari R, Salminen S, Palva A, de Vos WM: Comparative
454 genomic and functional analysis of *Lactobacillus casei* and
455 *Lactobacillus rhamnosus* strains marketed as probiotics. *Appl*
456 *Environ Microbiol* 2013;79:1923-1933.
- 457 Estrela S, Trisos CH, Brown SP: From metabolism to ecology: cross-
458 feeding interactions shape the balance between polymicrobial
459 conflict and mutualism. *Am Nat* 2012;180:566-576.
- 460 Floch MH, Walker WA, Sanders ME, Nieuwdorp M, Kim AS, Brenner DA,
461 Qamar AA, Miloh TA, Guarino A, Guslandi M, Dieleman LA,
462 Ringel Y, Quigley EMM, Brandt LJ: Recommendations for
463 probiotic use-2015 update proceedings and consensus opinion.
464 *J Clin Gastroenterol* 2015;49:S69-S73.
- 465 Forssten SD, Bjorklund M, Ouwehand AC: *Streptococcus mutans*, caries
466 and simulation models. *Nutrients* 2010;2:290-298.
- 467 Gruner D, Paris S, Schwendicke F: Probiotics for managing caries and
468 periodontitis: systematic review and meta-analysis. *J Dent*
469 2016;48:16-25.

470 Guggenheim B, Giertsen E, Schupbach P, Shapiro S: Validation of an in
471 vitro biofilm model of supragingival plaque. J Dent Res
472 2001;80:363-370.

473 Gupta P, Gupta N, Pawar AP, Birajdar SS, Natt AS, Singh HP: Role of
474 sugar and sugar substitutes in dental caries: a review. ISRN Dent
475 2013;2013:519421.

476 Hedberg M, Hasslof P, Sjostrom I, Twetman S, Stecksén-Blicks C: Sugar
477 fermentation in probiotic bacteria - an in vitro study. Oral
478 Microbiol Immunol 2008;23:482-485.

479 Hibberd PL, Kleimola L, Fiorino AM, Botelho C, Haverkamp M,
480 Andreyeva I, Poutsika D, Fraser C, Solano-Aguilar G, Snyderman
481 DR: No evidence of harms of probiotic *Lactobacillus rhamnosus*
482 GG ATCC 53103 in healthy elderly-a phase I open label study to
483 assess safety, tolerability and cytokine responses. PLoS One
484 2014;9:e113456.

485 Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L,
486 Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME: The
487 International Scientific Association for Probiotics and Prebiotics
488 consensus statement on the scope and appropriate use of the
489 term probiotic. Nat Rev Gastro Hepat 2014;11:506-514.

490 Jiang Q, Stamatova I, Kainulainen V, Korpela R, Meurman JH:
491 Interactions between *Lactobacillus rhamnosus* GG and oral
492 micro-organisms in an in vitro biofilm model. BMC Microbiol
493 2016;16:149.

494 Jiang Q, Stamatova I, Kari K, Meurman JH: Inhibitory activity in vitro of
495 probiotic lactobacilli against oral *Candida* under different
496 fermentation conditions. Benef Microbes 2015;6:361-368.

497 Jorgensen MR, Castiblanco G, Twetman S, Keller MK: Prevention of
498 caries with probiotic bacteria during early childhood. Promising
499 but inconsistent findings. Am J Dent 2016;29:127-131.

500 Laleman I, Detailleur V, Slot DE, Slomka V, Quirynen M, Teughels W:
501 Probiotics reduce mutans streptococci counts in humans: a
502 systematic review and meta-analysis. Clin Oral Investig
503 2014;18:1539-1552.

504 Laleman I, Teughels W: Probiotics in the dental practice: a review.
505 Quintessence Int 2015;46:255-264.

506 Lemos JA, Abranches J, Koo H, Marquis RE, Burne RA: Protocols to study
507 the physiology of oral biofilms. Methods Mol Biol 2010;666:87-
508 102.

509 Li J, Helmerhorst EJ, Leone CW, Troxler RF, Yaskell T, Haffajee AD,
510 Socransky SS, Oppenheim FG: Identification of early microbial
511 colonizers in human dental biofilm. J Appl Microbiol
512 2004;97:1311-1318.

513 Marsh PD: Are dental diseases examples of ecological catastrophes?
514 Microbiology 2003;149:279-294.

515 Marttinen A, Haukioja A, Karjalainen S, Nylund L, Satokari R, Ohman C,
516 Holgersson P, Twetman S, Soderling E: Short-term consumption

517 of probiotic lactobacilli has no effect on acid production of
 518 supragingival plaque. Clin Oral Investig 2012;16:797-803.
 519 Matsumoto M, Tsuji M, Sasaki H, Fujita K, Nomura R, Nakano K, Shintani
 520 S, Ooshima T: Cariogenicity of the probiotic bacterium
 521 *Lactobacillus salivarius* in rats. Caries Res 2005;39:479-483.
 522 Mayanagi G, Igarashi K, Washio J, Takahashi N: pH response and tooth
 523 surface solubility at the tooth/bacteria interface. Caries Res
 524 2017;51:160-166.
 525 Meurman JH, Antila H, Korhonen A, Salminen S: Effect of *Lactobacillus*
 526 *rhamnosus* strain GG (ATCC 53103) on the growth of
 527 *Streptococcus sobrinus* in vitro. Eur J Oral Sci 1995;103:253-258.
 528 Moye ZD, Zeng L, Burne RA: Fueling the caries process: carbohydrate
 529 metabolism and gene regulation by *Streptococcus mutans*. J
 530 Oral Microbiol 2014;6.
 531 Näse L, Hatakka K, Savilahti E, Saxelin M, Pönkä A, Poussa T, Korpela R,
 532 Meurman JH: Effect of long-term consumption of a probiotic
 533 bacterium, *Lactobacillus rhamnosus* GG, in milk on dental caries
 534 and caries risk in children. Caries Res 2001;35:412-420.
 535 Pande S, Kaftan F, Lang S, Svatos A, Germerodt S, Kost C: Privatization of
 536 cooperative benefits stabilizes mutualistic cross-feeding
 537 interactions in spatially structured environments. ISME J
 538 2016;10:1413-1423.
 539 Pham LC, Hoogenkamp MA, Exterkate RA, Terefework Z, de Soet JJ, ten
 540 Cate JM, Crielaard W, Zaura E: Effects of *Lactobacillus*
 541 *rhamnosus* GG on saliva-derived microcosms. Arch Oral Biol
 542 2011;56:136-147.
 543 Pham LC, van Spanning RJM, Roling WFM, Prosperi AC, Terefework Z,
 544 Ten Cate JM, Crielaard W, Zaura E: Effects of probiotic
 545 *Lactobacillus salivarius* W24 on the compositional stability of
 546 oral microbial communities. Arch Oral Biol 2009;54:132-137.
 547 Quevedo B, Giertsen E, Zijng V, Luthi-Schaller H, Guggenheim B,
 548 Thurnheer T, Gmur R: Phylogenetic group- and species-specific
 549 oligonucleotide probes for single-cell detection of lactic acid
 550 bacteria in oral biofilms. BMC Microbiol 2011;11:14.
 551 Salaun F, Mietton B, Gaucheron F: Buffering capacity of dairy products.
 552 Int Dairy J 2005;15:95-109.
 553 Sato Y, Yamagishi J, Yamashita R, Shinozaki N, Ye B, Yamada T,
 554 Yamamoto M, Nagasaki M, Tsuboi A: Inter-individual differences
 555 in the oral bacteriome are greater than intra-day fluctuations in
 556 individuals. PLoS One 2015;10.
 557 Schneider CA, Rasband WS, Eliceiri KW: NIH Image to ImageJ: 25 years
 558 of image analysis. Nat Methods 2012;9:671-675.
 559 Schwendicke F, Dorfer C, Kneist S, Meyer-Lueckel H, Paris S: Cariogenic
 560 effects of probiotic *Lactobacillus rhamnosus* GG in a dental
 561 biofilm model. Caries Res 2014a;48:186-192.
 562 Schwendicke F, Horb K, Kneist S, Dorfer C, Paris S: Effects of heat-
 563 inactivated *Bifidobacterium* BB12 on cariogenicity of

564 *Streptococcus mutans* in vitro. Arch Oral Biol 2014b;59:1384-
565 1390.

566 Selwitz RH, Ismail AI, Pitts NB: Dental caries. Lancet 2007;369:51-59.

567 Simark-Mattsson C, Emilson CG, Håkansson EG, Jacobsson C, Roos K,
568 Holm S: *Lactobacillus*-mediated interference of mutans
569 streptococci in caries-free vs. caries-active subjects. Eur J Oral
570 Sci 2007;115:308-314.

571 Snyderman DR: The safety of probiotics. Clin Infect Dis 2008;46 Suppl
572 2:S104-111; discussion S144-151.

573 Soderling EM, Marttinen AM, Haukioja AL: Probiotic lactobacilli interfere
574 with *Streptococcus mutans* biofilm formation in vitro. Curr
575 Microbiol 2011;62:618-622.

576 Stamatova I, Meurman JH, Kari K, Tervahartiala T, Sorsa T, Baltadjieva
577 M: Safety issues of *Lactobacillus bulgaricus* with respect to
578 human gelatinases in vitro. FEMS Immunol Med Microbiol
579 2007;51:194-200.

580 Takahashi N, Nyvad B: The role of bacteria in the caries process:
581 ecological perspectives. J Dent Res 2011;90:294-303.

582 Tanzer JM, Chassy BM, Krichevsky MI: Sucrose metabolism by
583 *Streptococcus mutans*, SL-I. Biochim Biophys Acta
584 1971;261:379-387.

585 Tehrani MH, Akhlaghi N, Talebian L, Emami J, Keyhani SE: Effects of
586 probiotic drop containing *Lactobacillus rhamnosus*,
587 *Bifidobacterium infantis*, and *Lactobacillus reuteri* on salivary
588 *Streptococcus mutans* and *Lactobacillus* levels. Contemp Clin
589 Dent 2016;7:469-474.

590 Thurnheer T, Gmur R, Guggenheim B: Multiplex FISH analysis of a six-
591 species bacterial biofilm. J Microbiol Methods 2004;56:37-47.

592 Thurnheer T, van der Ploeg JR, Giertsen E, Guggenheim B: Effects of
593 *Streptococcus mutans* gtfC deficiency on mixed oral biofilms in
594 vitro. Caries Res 2006;40:163-171.

595 Wolcott R, Costerton JW, Raoult D, Cutler SJ: The polymicrobial nature
596 of biofilm infection. Clin Microbiol Infect 2013;19:107-112.

597 Wu CC, Lin CT, Wu CY, Peng WS, Lee MJ, Tsai YC: Inhibitory effect of
598 *Lactobacillus salivarius* on *Streptococcus mutans* biofilm
599 formation. Mol Oral Microbiol 2015;30:16-26.

600 Xiao C, Ran S, Huang Z, Liang J: Bacterial diversity and community
601 structure of supragingival plaques in adults with dental health
602 or caries revealed by 16S pyrosequencing. Front Microbiol
603 2016;7:1145.

604 Yamada T, Takahashi-Abbe S, Abbe K: Effects of oxygen on pyruvate
605 formate-lyase in situ and sugar metabolism of *Streptococcus*
606 *mutans* and *Streptococcus sanguis*. Infect Immun 1985;47:129-
607 134.

608

609 **Legends**

610 **Table 1. Strains and the growth conditions.**

611 **Figure 1. Viable cell number of LGG from 64.5h multi-species**

612 **experimental oral biofilms.** Biofilms (5SP+LGG) were cultured with
613 fructose (BM-fructose), glucose (BM-glucose), lactose (BM-lactose),
614 sorbitol (BM-sorbitol), sucrose (BM-sucrose), and with carbohydrate
615 free (BM-negative) culture media. Three independent experiments were
616 conducted, each experiment contained two parallels. Every two parallels
617 generates an average. Three averages were involved in the statistical
618 analyse. Each average was based on log10 transformation, and analysed
619 with one-way ANOVA and Dunnett's test were compared with BM-
620 negative. Data represent the means \pm SDs, ***P<0.001.

621 **Figure 2. The pH of spent culture media for multi-species**

622 **experimental oral biofilms with (5SP+LGG) and without (5SP)**

623 **LGG.** pH was measured at 16.5, 40.5, and 64.5h. Three independent
624 experiments were performed, each experiment contained two parallels.
625 Two parallels generated an average. Three averages were involved in the
626 statistical analysis. One-way ANOVA with Duncan's test were done,
627 different small letters stand for a significant difference (P<0.05). Data
628 represent the means \pm SDs.

629 **Figure 3. FISH staining of fixed 64.5h biofilms of group 5SP+LGG**

630 **and 5SP cultivated in different carbohydrates.** The groups and tested

631 carbohydrates are labelled to the top left corner of each image. Green
632 (Lcas467-Cy3): LGG, red (MUT590-Cy5): *S. mutans*, blue (Hoechst):
633 the rest of the strains=*S. sanguinis* + *C. albicans* + *A.*
634 *actinomycetemcomitans* + *F. nucleatum*, pink/purple: co-localization of
635 red and blue, black: non-cells area. Column A: Group 5SP+LGG with
636 only green, column B: Group 5SP+LGG with only red and blue, column
637 C: Group 5SP+LGG with red and blue. Each image includes the
638 maximum intensity projections of xy- (top left), yz- (top right, rightmost
639 is closer to HA discs), and xz-planes (bottom, bottom end is closer to HA
640 discs). The scale bar is 30 μ m.

641 **Figure 4. Growth curves of LGG cultivated in biofilm broth media**
642 **with a sole carbohydrate for 48h.** The biofilm culture medium was
643 biofilm medium with fructose (BM-fructose), glucose (BM-glucose),
644 lactose (BM-lactose), sorbitol (BM-sorbitol), sucrose (BM-sucrose), or
645 with carbohydrate free (BM-negative). Two independent experiments
646 were performed, each experiment contained three parallels. Data
647 represent the means \pm SDs of all six values.